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Coordinatore: Prof. Stefania Montagnani

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CD117-POSITIVE CARDIAC PRIMITIVE CELLS: THEIR LOCALIZATION AND INTERACTION WITH EXTRACELLULAR MATRIX IN THE HUMAN ADULT NORMAL AND PATHOLOGICAL HEART

Relatore:

Prof. Stefania Montagnani

Candidata:

Dott. Daria Anna Nurzynska

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INDEX

ABSTRACT

CD117-positive cells contributing to the cardiac cell turnover in the normal and pathological conditions have been recently described in the adult human heart. Since the precise spatial and temporal expression of extracellular matrix proteins and their receptors is critical for proper organ formation, we have analyzed and compared the spatial distribution of cardiac primitive CD117-positive cells in the human adult normal and pathological hearts with chronic ischemic cardiomyopathy, with respect to the localization and expression of laminin and integrin isoforms.

In the pathological hearts, CD117-positive cells, visualized by immunofluorescence, were significantly more numerous than in the normal hearts. These cells were localized mainly in the atria and were up to 38-fold more numerous in the subepicardium than in the myocardium. Compared with normal hearts, most CD117-positive cells in the subepicardium of pathological hearts were α_6 integrin-positive. Laminin-1, typical of developing heart, was found predominantly in the subepicardium of normal and pathological adult hearts. Western blotting revealed its highest expression in the normal atrium and pathological left ventricle. The presence of laminin-1 *in vitro*, in contrast to laminin-2, increased proliferation and reduced apoptosis of CD117-positive cells, with the latter effect being related to integrin α_6 expression. *In vitro*, the epithelial-mesenchymal transition of epicardial cells from human adult heart, cultured

in the presence of extracellular matrix synthesized by cardiac fibroblasts, gave origin to CD117-positive cells.

These data reveal that the increase in the number of cardiac CD117-positive cells and the expression of laminin-1 are observed in the ischemic cardiomyopathy. The subepicardial localization of CD117-positive cells, laminin-1 and α_6 integrin subunit expression may all correspond to the activation of cardiac regeneration involving an epithelial-mesenchymal transition recently described also in the adult heart.

1. Introduction

1.1. CD117-positive cells as cardiac stem cells

Stem cells and progenitor cells with the capacity to differentiate into three major cardiac cell types - cardiomyocytes, smooth muscle cells and endothelial cells have been described in both embryonic [1, 2, 3] and adult heart tissue [4, 5, 6]. These cells have been characterized by an array of membrane, cytoplasmic and nuclear antigens, yet the expression of different markers could be associated simply with the degree of stem cell differentiation. Despite different phenotype description in various studies, it is widely acknowledged that the cardiac hematopoietic lineage-negative (CD45-negative, CD34-negative) stem cells express neither muscle nor endothelial cell markers at an undifferentiated stage [7]. However, when induced to differentiate, they adopt phenotype specific for myocytes, smooth muscle or endothelial cells, expressing markers typical for these cell lineages (Nkx2.5 and α-sarcomeric actin, GATA6 and smooth muscle actin, Ets-1 and FVIII, respectively).

Although the specification and origin of cardiac stem cell population remains to be determined, many studies have reported the presence of hematopoietic lineage-negative, stem cell factor receptor (CD117)-positive primitive cells with the above characteristics in the adult myocardium [4, 5, 8]. In the light of those findings, it is reasonable to suppose that the heart is

not a terminally differentiated organ and cardiac cells are continuously replaced by newly formed younger populations of myocytes as well as vascular smooth muscle and endothelial cells. These cellular processes are enhanced in pathologic states. However, the precise mechanisms controlling primitive cardiac cells (defined as cells expressing stem cell markers only or together with markers of commitment towards cardiac cell lineages) survival, proliferation and migration in the diseased heart need to be elucidated further.

1.2. Laminin isoforms and their receptors in the heart

It is known that the precise spatial and temporal expression of extracellular matrix proteins and their receptors is critical for proper organ formation during organogenesis [9]. Laminin-1 (heterotrimer assembled from α1, β1 and γ1 chain subunits) is the first extracellular matrix protein to be expressed during embryonic development and it has been observed that the heart organogenesis does not proceed in the absence of this protein [10]. The absence of laminin-2 (α2β1γ1), an isoform typical of the muscle, causes congenital muscular dystrophy with cardiac involvement [11]. Similarly, the presence of specific integrins influences the fate and biological properties of cells [12]. A correct interaction of extracellular matrix proteins with their receptors on the cell membrane and outside-inside integrin signaling has been found to be critical in the rod shape formation of primitive myocytes

and patterning of the myofibrils in vitro [13]. Receptors for laminin-1 and -2 present on adult cardiomyocytes include integrins $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_7\beta_1$, whereas $\alpha_6\beta_4$ is typically found on epithelial cells [14]. Although a primary function of $\alpha_6\beta_4$ integrin is to maintain the integrity of epithelia due to its ability to mediate the formation of hemidesmosomes on the basal cell surface [15], some studies provide definitive evidence to implicate $\alpha_6\beta_4$ in migration, documenting its localization in membrane protrusions associated with migration [16]. Importantly, the acquisition of motile properties by epithelial cells can be a consequence of an epithelial-mesenchymal transition [17].

1.3. Epithelial-mesenchymal transition of epicardial cells

Epithelial-mesenchymal transition is one of the major events in heart organogenesis. Studies of developing avian embryos have shed light on the mechanisms by which cardiac cells are formed from the proepicardium [18]. In mammals, the proepicardium is composed of mesothelium continuous with splanchnopleural epithelium that separates the primitive pleural/pericardial cavity from the peritoneal cavity. The epithelial cells of proepicardium attach to the surface of the developing myocardium, forming epicardium. Subsequently, a subset of epicardial cells undergoes an epithelial-mesenchymal transition (EMT), that is they detach from the epithelial sheet, invade the subepicardial space and acquire a mesenchymal

phenotype, generating in the subepicardium the population of epicardially derived cells (EPDCs) [19]. The epithelial-mesenchymal transition is triggered by interplay of extracellular matrix proteins and growth factors and requires dissociation of tight junctions, acquisition of motile properties and molecular changes involving epithelial and mesenchymal markers expression [17]. EPDCs differentiate into interstitial fibroblasts, cardiomyocytes, coronary endothelial and smooth muscle cells [20]. Recent studies have observed the preservation of vasculogenic potential of adult epicardial cells *in vitro* [21, 22], but the possibility that these cells are the real cardiac stem cells of the human heart awaits investigation.

2. Scope of the study

The adult heart is mainly composed of terminally differentiated cells, but it is not a terminally differentiated organ since it harbors stem cells supporting its regeneration. Self-renewing organs contain the niches that constitute the microenvironment in which primitive cells survive, proliferate, differentiate and from which they migrate upon stimulation. The structure of a niche needs to suit the particular needs of its resident stem cells [23]. Hence, the scope of the study was to observe cardiac primitive cells, their microenvironment and their interactions mediated by integrins, in the normal and pathological conditions.

To this aim, the spatial distribution of CD117-positive cells in the adult human normal and pathological hearts with chronic ischemic cardiomyopathy was analyzed and compared with respect to the localization and expression of laminin-1 and laminin-2, and their receptor, integrin $\alpha_6\beta_4$. Furthermore, the role of laminin-1 and laminin-2 in the primitive adult cardiac cell proliferation and apoptosis was examined *in vitro*.

The description of cardiac primitive cells in the adult human heart stimulated a heated and fierce debate about their origin, dividing basic science and clinical researchers community. In fact, most studies attempting to cast new light on the characteristics of CD117-positive cells and the mechanisms controlling their homing, migration and differentiation, raise more questions rather than provide definitive answers. Also in the course of

our study, the findings resulting from the description of CD117-positive cells localization and extracellular matrix interactions dictated the need for subsequent research on the origin of cardiac primitive cells in the adult human heart. Hence, the possibility that epithelial-mesenchymal transition of epicardial cells can result in the generation of CD117-positive cells in the adult human heart was examined *in vitro*.

3. Materials and methods

3.1. Materials

Samples of normal adult hearts were derived from patients who died for reasons other than cardiovascular disease (n=11, mean age 41±12 years, 7 males, 4 females). Pathological hearts were explanted due to end-stage heart failure associated with ischemic cardiomyopathy (n=20, mean age 55±5.5 years, 14 males, 6 females, mean ejection fraction 25±1%). In each case fragments of right ventricle, left atrium, left atrioventricular junction, left ventricle and apex were excised across the full thickness of heart wall, including epicardium and endocardium. The investigation conforms with the principles outlined in the Declaration of Helsinki.

3.2. Immunofluorescence

3.2.1 Immunofluorescent staining of heart tissue sections

Heart tissue was embedded in Killik cryostat embedding medium (Bio-Optica, Milan, Italy), fast frozen and stored at -80°C or fixed in 4% formaldehyde and embedded in paraffin, then sliced into serial 4 μm-thick sections and mounted on slides. Before proceeding for immunostaining, the paraffin was removed by incubation with xylen, followed by rehydration

with ethanol at decreasing concentrations (from 96 to 80% v/v). For cellular antigens retrieval, sections immersed in citric buffer were heated in microwave oven. Frozen sections were fixed in 4% paraformaldehyde and equilibrated to room temperature, then dehydrated with ethanol at increasing concentrations (from 30 to 95% v/v). Slides were incubated in blocking buffer and double labeling was performed using primary antibodies against CD117 (1:10; Dako, Glostrup, Denmark) and α_6 integrin (1:20; Santa Cruz Biotechnology, Santa Cruz, CA, USA); fibronectin (1:200; Sigma-Aldrich, St. Louis, MO, USA) and α_4 integrin (1:20); laminin α-1 (1:25) or α-2 chain (1:25; all from Santa Cruz Biotechnology) and α-sarcomeric actin (1:30; Sigma-Aldrich); and secondary antibodies conjugated with fluorescein or rhodamine (1:30; Jackson Immunoresearch, West Grove, PA, USA). Nuclei were counterstained with DAPI. Finally, sections were covered with mounting solution (Vector Laboratories, Burlingame, CA, USA) and coverslip. Negative controls were included for each staining by elimination of primary or secondary antibody. Microscopic analysis was performed with a Leica DMLB microscope equipped for epifluorescence. For every field, images corresponding to different immunofluorescence filters were taken with digital camera connected to a microscope (Leica DC200) and then merged (Leica QFluoro). The number of CD117-positive cells/100 mm^2 was established by counting all positive cells in the section and measuring the area of the entire section (SigmaScan Pro5 software).

3.2.2. Immunofluorescent staining of cardiac cells *in vitro*

For the characterization of cells *in vitro*, CD117-positive cells were fixed with 4% paraformaldehyde. After incubation in blocking serum, nuclear and cytoplasmic markers of different cardiac cell lineages were labeled with primary antibodies against CD117 (1:10, Dako), Nkx 2.5 (1:20) and α-sarcomeric actin (1:30), Ets-1 (1:40) and factor VIII (1:40), GATA-6 (1:40; all from Santa Cruz Biotechnology) and smooth muscle actin (1:30; Sigma-Aldrich) and secondary antibodies conjugated with fluorescein or rhodamine (1:30; Jackson Immunoresearch). Nuclei were counterstained with DAPI and the cells were mounted under a glass coverslip. Images corresponding to different immunofluorescence filters were taken with digital camera (Leica DC200) connected to a microscope (Leica DMLB) and then merged (Leica QFluoro).

3.3. Immunoprecipitation and immunoblotting

Protein extracts were prepared from fragments of normal and pathological hearts. Heart tissue (1 gram) was minced and incubated on ice in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100 supplemented with proteases inhibitors (1 mM DTT, 2 mM PMSF, 2 μg/ml aprotinin and 10 μg/ml leupeptin). Lysates were centrifuged and protein concentration in the supernatants was

determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Solutions containing 300 μg of proteins were incubated with antibody against laminin α_1 or α_2 chains (1:10; Santa Cruz Biotechnology), followed by the incubation with Protein G Agarose Beads (Invitrogen, Carlsbad, CA, USA). Western blot of α–actinin (1:100; Santa Cruz Biotechnology) served as a control of equal protein amount in the solution used for immunoprecipitation. The immunoprecipitated proteins were size fractionated by electrophoresis on 8% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Molecular weight markers (Bio-Rad Laboratories) and denaturated samples of purified laminin were loaded onto each gel as a molecular weight and positive immunoblotting control, respectively. The membranes were blocked and then incubated with anti-β_1 laminin antibody (1:100), followed by horseradish peroxidase-labeled secondary IgG (1:10000; both from Santa Cruz Biotechnology). Antibody binding was visualized by chemiluminescence (GE Healthcare, Bucks, UK) and autoradiography. The intensity of individual bands was determined using ImageJ software (NIH, USA).

3.4. Cardiac primitive cell isolation and culture

3.4.1. Establishment of primary cardiac cell culture

For *in vitro* assays, CD117-positive cells were isolated from the fragments of left ventricular myocardium of pathological hearts. The primary culture was obtained according to the protocol described previously [24] with some modifications. Cardiac tissue fragments were minced and digested in the presence of 0.25% trypsin-EDTA and collagenase II (0.1% w/v). Cardiomyocytes were removed by sequential centrifugation and the supernatant was filtered with 40 μm nylon cell strainer. The cells were plated at the density of $2x10^4/cm^2$ in DMEM-Ham F12 medium (Sigma-Aldrich) supplemented with 5% fetal calf serum (Invitrogen), bFGF (10ng/ml, Peprotech, London, UK), glutathione (0.2mM, Sigma-Aldrich), penicillin G (50,000U) and streptomycin (50mg, Invitrogen) and allowed to proliferate.

3.4.2. Isolation of CD117-positive cells

Once adherent cells were more than 75% confluent, they were detached with 0.25% trypsin-EDTA and CD117-positive cells were purified using positive selection with anti human-CD117 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as recommended by the

manufacturer. Briefly, cells were incubated with magnetically labeled monoclonal anti-human CD117 antibodies (1:5) and FcR blocking reagent (1:5). Then, the cell suspension was loaded onto a column that was placed in the magnetic field. While the unlabeled cells ran through, the magnetically labeled CD117-positive cells were retained on the column and eluted as the positively selected fraction once the column had been removed from the magnetic field.

3.5. Culture of cardiac primitive cells in the presence of laminin-1 and -2

To evaluate the effects of laminin-1 and laminin-2 on cardiac primitive cells proliferation and apoptosis, CD117-positive cells isolated from adult human heart were cultured on laminin-1 (R&D Systems, Minneapolis, MN, USA) or laminin-2 (Sigma-Aldrich) coated dishes (5μg/cm^2). In order to assess the role of α_6 integrin signaling in the regulation of these processes, before *in vitro* testing, cells were detached, divided into three groups and incubated in suspension with function blocking anti-α_6 integrin antibody (Chemicon, Temecula, CA, USA) or isotype-matched control antibody for 1 hour. Third group of cells was processed in the same manner but without antibody presence. Next, the cells were seeded on chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) coated with either laminin-1 or laminin-2. Chamber slides covered with

bovine serum albumin (BSA, Sigma-Aldrich) served as control. All *in vitro* experiments were repeated a minimum of three times in triplicate.

3.5.1. Evaluation of proliferation on laminin-1 and -2 *in vitro*

For evaluation of proliferation, cardiac primitive cells were exposed to serum-free culture medium for 12 hours. Quiescent cells were incubated with complete medium for 24 hours and 5-bromo-2'-deoxy-uridine (BrdU) was added (10μM) for one hour before cell fixation. BrdU is incorporated in place of thymidine during DNA synthesis. Incorporation of BrdU was evaluated using BrdU Labeling and Detection Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The cells were fixed in acidic ethanol and incubated with anti-BrdU monoclonal antibody (1:10) in incubation buffer containing nucleases. After incubation with anti-mouse-Ig-fluorescein (1:40), the nuclei were counterstained with DAPI and the signals were visualized by immunofluorescence microscopy. The result was expressed as the percentage of BrdU-positive cells.

3.5.2. Evaluation of apoptosis on laminin-1 and -2 *in vitro*

For evaluation of apoptosis, cardiac primitive cells were cultured on laminin-1 or laminin-2 for 24 hours and then fixed in 1% paraformaldehyde. Apoptotic cells were labeled using ApopTag Plus Fluorescein *In Situ*

Apoptosis Detection Kit according to the manufacturer's protocol (Chemicon). DNA strand breaks were detected by labeling the 3'-OH termini generated upon apoptotic DNA fragmentation with modified, digoxigenin labeled nucleotides enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). Incorporated nucleotides were then allowed to bind an anti-digoxigenin antibody conjugated to fluorescein. All nuclei were counterstained with DAPI. The apoptotic cells were quantified by fluorescence microscopy. The result was expressed as the percentage of apoptotic cells.

3.6. Epicardial cell culture

3.6.1. Formation of extracellular matrix substrate *in vitro*

Cardiac fibroblasts were isolated from the fragments of adult human pathological hearts using positive selection with anti-fibroblast MicroBeads (Miltenyi Biotec) as recommended by the manufacturer. Cells were incubated with magnetically labeled monoclonal anti-fibroblast antibodies and loaded onto a column placed in the magnetic field. Successively, the positive fraction was eluted from the column once it had been removed from the magnetic field. The fibroblasts were plated at the density of 30 $x10^3$ cells/cm^2 in DMEM with 10% fetal calf serum. After one week cells were non-enzymatically removed with delicate repetitive washes in 0.5mM

EDTA and the presence of extracellular matrix proteins on the culture dish was confirmed by immunofluorescence.

3.6.2. Epithelial sheet formation *in vitro*

Extracellular matrix synthesized and secreted by the fibroblasts isolated from human adult heart served as a substrate for the culture of epithelial cells from the fragment of epicardium detached mechanically from the heart wall. The fragments were placed on culture dishes layered with extracellular matrix in the medium composed of DMEM-Low Glucose (Invitrogen) and MCDB 201 (US Biological, Swampscott, MA, USA) supplemented with 2% fetal bovine serum (Invitrogen), Linoleic Acid-BSA (1mg/ml), ITS 100X, Ascorbic Acid-2-Phosphate (0.1mM, all from Sigma-Aldrich), penicillin G (50,000U) and streptomycin (50mg, Invitrogen). After a period ranging from 3 to 5 days the outgrowth of epithelial cells was observed *in vitro*.

3.7. Epithelial-mesenchymal transition of epicardial cells *in vitro*

To evaluate whether epithelial-mesenchymal transition in the adult human heart gives rise to CD117-positive cells, hepatocyte growth factor (HGF, 40ng/ml, Peprotech) was added to the culture of cardiac mesothelial cells forming an epithelial sheet on the dish layered with extracellular

matrix substrate. The effects of HGF were observed as soon as 24 hours later. After 48 hours the cells were fixed and stained with anti-CD117 antibody (Dako), as described above.

3.8. Statistics

Quantitative results are expressed as means±SEM. Statistical differences were evaluated using Student's two-tailed *t*-test for comparison among pairs of groups and ANOVA with post-hoc Bonferroni's *t*-test when multiple groups were compared. *P* values <0.05 were considered statistically significant.

4. Results

4.1. Quantification of CD117-positive cells in the adult human heart

4.1.1. Distribution of CD117-positive cells between different heart regions

The presence of CD117-positive cells was analyzed by immunofluorescence (fig. 1A, E). In the normal hearts, there were 345.5±32.2 cells/100 mm^2 in the right ventricle, 409.6±11.6 cells/100 mm^2 in the left atrium, 126.7±3.7 cells/100 mm^2 in the atrioventricular junction, 248.0±67.3 cells/100mm^2 in the left ventricle and 105.7±33.3 cells/100 mm^2 in the apex (fig. 2). In the hearts with chronic ischemic cardiomyopathy CD117-positive cells were significantly more numerous than in the normal hearts. Interestingly, their distribution between different heart regions did not differ significantly and also in the pathological hearts it was the left atrium that contained more CD117-positive cells (3573.0±874.9 cells/100 mm^2), followed by the left ventricle (3243.0±1134.0 cells/100 mm^2) and the right ventricle (2809.0±715.3 cells/100 mm^2). The atrioventricular junction and the apex contained 465.3±57.6 and 575.8±60.2 cells/100 mm^2, respectively (fig. 2).

4.1.2. Distribution of CD117-positive cells between different tissue layers

Both in the normal and in the pathological hearts, CD117-positive cells were strikingly more numerous within the epicardium and subepicardium than in the main myocardium. In the normal hearts (fig. 3A), this difference reached a maximum of 27.5-fold in the left atrium (14.3±1.6 cells/100mm^2 in the myocardium and 395.2±11.7 cells/100mm^2 in the epicardium, p<0.0001) and a minimum of 6.7-fold in the apex (13.75±2.9 cells/100mm^2 in the myocardium and 91.9±30.8 cells/100mm^2 in the epicardium, p<0.05). In the hearts with ischemic cardiomyopathy (fig. 3B), the subepicardium within the left atrium contained 38-fold more CD117-positive cells than the main myocardium (3483.3±870.8 versus 91.9±5.2 cells/100mm^2, p<0.0001). Similarly, there were 10.5-, 42-, 8- and 37.5-fold more CD117-positive cells in the subepicardium compared with the main myocardium of the atrioventricular junction (424.9±59.3 vs. 40.4±2.6 cells/100mm^2, p<0.0001), left ventricle (3188.1±1126.0 vs. 56.6±5.2 cells/100mm^2, p<0.005), apex (512.5±57.4 vs. 63.3±4.6 cells/100mm^2, p<0.0001) and right ventricle (2624.8±612.8 vs. 69.8±5.5 cells/100mm^2, p<0.0001), respectively.

It emerges that the increase in the number of CD117-positive cells in the chronic pathological conditions is more pronounced within the subepicardium than in the myocardium of all heart regions. Compared with normal hearts, the number of CD117-positive cells raises 10-fold in the

subepicardium and only 5-fold in the myocardium of the left ventricle in the hearts with ischemic cardiomyopathy.

4.2. Integrin α_6 expression on CD117-positive cardiac cells

A fraction of CD117-positive cells expressed α_6 integrin subunit (fig. 1B), which together with β_4 constitutes a typical and specific receptor for laminin. Examination of heart sections by immunofluorescence showed that this fraction was almost identical in the subepicardium (19.51±5.6%) and myocardium (21.67±3.48%, *p* value ns) of the normal hearts (fig. 3A). In the pathological hearts (fig. 3B), by contrast, α_6 integrin-expressing cells constituted 82.73±9.1% of CD117-positive cells in the subepicardium and only 40.7±17% of CD117-positive cells in the myocardium ($p<0.05$) of the left atrium, and 86.12±9.4% in the subepicardium and 65.44±3.45% in the myocardium of the left ventricle ($p<0.05$).

4.3. Laminin isoforms expression in the adult human heart

Laminin α_1 chain makes part of laminin-1 and -3, while α_2 laminin is present in laminin-2, -4 and -12 isoforms [25]. Of these, only laminin-2 and -4 (merosins) are present typically in the normal adult human heart, while laminin-1 is expressed in the developing myocardium [26]. Fluorescent labeling of heart cryo-sections with antibodies specific for α_1 and α_2 laminin

chains revealed the presence of laminin-1 ($\alpha_1\beta_1\gamma_1$) and merosins (α_2 chain containing laminins) in the subepicardium and myocardium of human adult heart (examples of left ventricular tissue staining are presented in fig.4). Protein analysis by electrophoresis and immunoblotting showed differences in the expression of laminin-1 and laminin-2 between different regions, as well as between the same regions of the normal and pathological hearts (fig.5 and 6).

4.3.1. Pattern of laminin-1 and -2 distribution in the heart tissue

In the normal hearts, laminin-1 presence was restricted to the epicardium and subepicardium, while α_2 laminin chain bordered, but not surrounded entirely, cardiomyocytes. In the hearts with ischemic cardiomyopathy, laminin-1 formed a meshed network within the subepicardium, with frequent inward-reaching branches (fig. 4A), and filled the interstitial space in the myocardium, revealing granular-like pattern of fluorescence around the cardiomyocytes (fig. 4B, C). Merosins lined the epicardium (fig. 4D) and formed a conspicuous network lining the basement membrane of the cells in the myocardium (fig. 4E, F).

4.3.2. Expression of laminin in different heart regions

While in the atria the expression of $\alpha_1\beta_1$ laminin chains (fig.5A), detected by immunoprecipitation and immunoblotting, was only slightly higher in the normal than in pathological hearts, in the left ventricle it was up to 3-fold higher ($p<0.001$) in the hearts with ischemic cardiomyopathy. However, the expression of laminin-1 in the normal atria was 4-fold higher than in the normal left ventricle ($p<0.001$), whereas in the pathological conditions it was the left ventricle that contained more laminin-1 than any other heart region (fig. 6).

Laminin-2 expression in the atria from normal and pathological hearts differed not significantly (fig.5B), whereas in the left ventricle $\alpha_2\beta_1$ chains were up to 2.5-fold more abundant ($p<0.001$) in the pathological hearts.

4.4. Culture of cardiac primitive cells in the presence of laminin-1 and -2 *in vitro*

4.4.1. Characterization of CD117-positive cells *in vitro*

CD117-positive cells were isolated from primary cardiac cell culture by immunomagnetic cell sorting (as described in Materials and Methods section 2.4.2). Purity of sorted cells was determined by immunofluorescence

and reached 98%. The culture consisted of CD117-positive cardiac lineage-negative cells and CD117-positive cells that expressed markers of commitment towards cardiac cell lineages (fig.7). Fluorescent immunolabeling revealed the presence of endothelial (expressing transcription factor Ets-1 in the nuclei and FVIII in the cytoplasm) and smooth muscle cells progenitors and precursors (expressing transcription factor GATA6 in the nuclei and smooth muscle actin in the cytoplasm), as well as cells committed to cardiomyocyte lineage (with transcription factor Nkx2.5 and α-sarcomeric actin fibers).

4.4.2. Proliferation and apoptosis in the presence of laminin-1 and -2 *in vitro*

To investigate the role of different laminin isoforms in the cardiac primitive cell proliferation and survival, CD117-positive cardiac primitive cells were isolated from the adult human heart and cultured on laminin-1 or laminin-2 coated chamber slides. In the presence of laminin-2 the proliferation rate of cells (fig. 6A), evaluated by the incorporation of BrdU *in vitro*, was 7.7±0.1% (n=4), whereas during the culture of cardiac primitive cells on laminin-1-coated dishes the proliferation rate increased more than 4-fold and reached 31.1±1.4% (n=4, $p<0.001$). Moreover, apoptosis in the presence of laminin-1 (fig. 6B) was 4-fold lower (1.1±0.2%, n=5) than in the culture of cells on laminin-2 (4.4±0.5%, n=5, $p<0.001$). This effect of laminin-1 was inhibited by earlier incubation of cells with α_6

integrin function-blocking antibody, implicating α_6 integrin and laminin-1 interaction in the regulation of CD117-positive cell survival.

4.5. Epithelial-mesenchymal transition of the adult epicardial cells *in vitro*

The fragments of epicardium placed on the culture dish covered with extracellular matrix proteins synthesized and secreted by cardiac fibroblasts gave rise to an epithelial sheet *in vitro*. The epithelial sheet was formed by a monolayer of polygonal cells tightly joined together (fig. 9A). The addition of HGF resulted in the spectacular change of cell morphology and as soon as 24 hours later, we observed small spindle-like shaped cells with numerous lamellipodia and motile properties (fig. 9B). The majority of cells expressed CD117 antigen characteristic of cardiac primitive cells (fig. 9C). Hence, the epithelial-mesenchymal transition of mesothelial cells from adult human epicardium gives rise to the cardiac primitive cells *in vitro*.

5. Discussion

5.1. Subepicardial localization of cardiac CD117-positive cells in the adult human heart

The space between the myocardium and epicardium is known as the subepicardium or subepicardial space. This compartment plays a specific and critical role in the heart development, as it hosts mesenchymal cells that detach from epithelial sheet of epicardium and generate a population of epicardially derived cells (EPDCs) through an epicardial epithelial-mesenchymal transition (EMT) [19]. Recent studies have demonstrated that EPDCs can differentiate into multiple cell types, including coronary endothelial and smooth muscle cells, as well as cardiac fibroblasts [20, 27] and the process in which epicardially-derived cells acquire mesenchymal phenotype and invade myocardium giving origin to cells of cardiac lineages has been recently suggested as the source of stem cells also in the adult heart [21, 22].

In the previous studies [6, 8, 28], it was shown that the population of CD117-positive cells reside in the adult heart. The quantification of cardiac primitive cells revealed that their pool was enhanced acutely after infarction, but this growth response was attenuated in chronic heart failure [29]. Moreover, the clusters of proliferating myocytes, smooth muscle end endothelial cells were found in acutely infarcted hearts, but not in chronic

infarcts. This is in agreement with the present study, in which we have found CD117-positive cells in the hearts with ischemic cardiomyopathy scattered within myocardium. However, we report the presence of numerous CD117-positive cells within epicardium and subepicardium of the adult human heart.

To the best of my knowledge, this is the first study showing that the increase in the number of CD117-positive cells between human normal and pathological hearts with ischemic cardiomyopathy involves predominantly the cells localized in the subepicardium, and to a lesser, although still significant, extent those within the myocardium. The subepicardial localization of CD117-positive cells seems to correspond to their likely origin, that is an epithelial-mesenchymal transition of epicardial cells.

A numeric increase of cardiac primitive cells in the adult human heart has already been described in the patients with chronic aortic stenosis [30]. In that study, increased number of c-kit positive cells expressing markers of differentiation towards the cells of cardiac lineages was found within the myocardium. It is possible that the epicardial and subepicardial localization of CD117-positive cells has not been taken into consideration or else, different type of pathological stress evokes different types of response, with the prevalent activation of differentiation and maturation of primitive cells resident within the myocardium in the pressure overload and the generation, proliferation and migration of CD117-positive cells within a subepicardial space in the chronic ischemia.

5.2. Integrin α_4 and α_6 expression on CD117-positive cardiac cells

Both different laminin isoforms and integrin subunits have different functions and convey specific signals within the cell. Moreover, integrins are associated with growth factor and cytokine receptors, coordinating the response of cells to the changes of the fibrillar and soluble components of extracellular matrix [12].

In the present study of the localization of CD117-positive primitive cells in the adult human heart, we found only sparse CD117-positive cells in the normal myocardium and numerous CD117-positive cells that expressed α_4 integrin within the epicardium lined with fibronectin. In contrast, the epithelial cells were absent from the surface of the heart with ischemic cardiomyopathy. Moreover, none of the CD117-positive cells localized within the myocardium expressed α_4 integrin. This is consistent with the results of Dettman et al. [31] who found that the receptor of fibronectin, α_4 integrin, normally restrains epicardial-mesenchymal transition, as well as invasion and migration of epicardially derived mesenchymal cells during organogenesis. Integrin α_4 has also been implicated in the epicardium development and integrity during embryogenesis [32, 33]. In the adult, this integrin subunit is typically present on differentiated cells of mesenchymal origin [34] and its interaction with interstitial fibronectin may enable functional integration of newly formed cardiac cells in the myocardial syncytium *in vivo*.

Urbanek et al. [35] described clusters of cardiac lineage-negative, CD117-positive, α_4 integrin-positive cells surrounded by fibronectin and laminin α_2 chain within the myocardium of normal adult mice. Far-reaching analogy with the bone marrow made them conclude that α_4 integrin – laminin-8/9, -10/11 and fibronectin interaction is implicated in cardiac stem cells renewal and the preservation of their undifferentiated state. This is a very plausible theory, however, it is based on erroneous assumption, since, to the best of my knowledge, there is no interaction of laminin with α_4 integrin or the presence of α_2 laminin chain in the laminin-8/9 or -10/11 isoforms [25].

In the present study, CD117-positive cells in the subepicardial space co-localized with laminin-1, lacked α_4 integrin and expressed α_6 integrin subunit. Apart from the classical role of integrin α_6 in the maintenance of cell adhesion, it has been described for several different cell types that, rather than being downregulated, this subunit becomes widely distributed in the cell membrane and participates in cell migration on laminin-1 [36]. In the light of these facts, both laminin-1 presence in the subepicardial space and α_6 integrin expression on cardiac CD117-positive cells in the adult heart, described in the present study, may enable the migration of cardiac primitive cells from the subepicardium towards the damaged muscle.

5.3. Laminin isoforms in the adult human heart

Laminin-1 predominates among the laminin forms during early embryogenesis and further organogenesis. Its unique role is underlined by the fact that the embryogenesis will not proceed in the absence of this form of laminin [10]. To the best of my knowledge, this is the first study documenting the presence of laminin-1 in the adult human heart. In the immunochemical study of the laminin content in embryonic and adult mouse tissues, Sasaki et al. [37] found laminin-2 expression in the normal adult murine heart and skeletal muscle to be the highest compared to all other organs, whereas the content of α_1 chain was much lower. While that study analyzed whole-organ tissue extracts, we focused on the differences in the laminin expression between different regions (mostly atrium and left ventricle) and different layers (subepicardium, myocardium) of the normal and pathological adult hearts.

We found that in the adult human hearts the presence of laminin α_1 chain, as detected by immunofluorescence, was restricted mostly to the subepicardial space, with the highest expression in the normal atria and pathological left ventricle. Laminin-1 is essential in epithelial tissue polarization, as well as in epithelial-mesenchymal contact and interactions [38]. These two functions may be essential also in the heart, particularly in the subepicardial space, that hosts CD117-positive cardiac cells. In the liver, an organ with high regenerative capacity in the adult life, laminin α_1 that

disappears from the space of Disse by 6 to 8 weeks of postnatal life, reappears during hepatic regeneration [39]. The expression of laminin-1 in the adult human heart, involving molecular reprogramming and revoking the mechanisms operative during organogenesis, may be directly associated with and aim at the regeneration of cardiac tissue in the chronic pathological conditions. The presence of laminin-1 in the subepicardial space of ischemic heart may be essential for creating an environment permissive for epithelial-mesenchymal transition in the adult heart.

It is known that the absence of, or alteration in the laminin α_2 chain weakens the muscle cells basement membrane, which leads to muscle fiber damage under the stress of contractions [40]. Whereas α_2 chain is expressed by differentiated mature cells of mesodermal origin, laminin α_1 chain is highly expressed by developing epithelial cells [37]. It follows that while the expression of laminin-1 isoform in the diseased heart may have important role in the formation of new functionally competent cells, laminin-2 expression would be essential for the maintenance of the pre-existing cardiac cells. Importantly, the differences in the laminin expression in the pathological hearts found and described in the present study, regarded mostly laminin α_1 chain with the preserved and even increased laminin α_2 expression in the ischemic myocardium when compared with the normal heart. Moreover, the atrium of the normal adult human hearts contained more laminin-1 than the left ventricle, while the increase of laminin-1 content in the pathological conditions regarded mostly the left ventricular

wall, subjected to the highest work overload in case of dilative ischemic cardiomyopathy.

The observation of CD117-positive cells *in vitro* indicate that laminin-1, not laminin-2, presence protects from apoptosis and stimulates proliferation of cardiac primitive cells. The α_6 integrin presence and function was not implicated in the proliferation of cells on laminin-1, but it diminished the apoptosis rate. This effect is similar to observations reported by Maitra et al. [34] in which myoblasts transfected with α_6 did not proliferate, but were able to differentiate. Our findings of the increased fraction of α_6 integrin expressing CD117-positive cells in the pathological human hearts with respect to the normal hearts may support the role of α_6 integrin-laminin interaction in the survival, migration and differentiation of cardiac primitive cells activated in the chronic pathological conditions.

5.4. Epithelial-mesenchymal transition in the adult human heart

The adult myocardium is covered by a layer of epithelial cells forming epicardium. Epicardial cells are involved in the earliest events during the establishment of myocardial tissue. These events were studied in detail in the chick and quail model, in which a proepicardial organ forms a separate structure that can be manipulated, microdissected and isolated for *in vitro* studies. In mammals, the proepicardium is composed of mesothelium derived from septum trasversum and continuous with

splanchnopleural epithelium. This mesothelium synthesizes a dense layer of extracellular matrix and develops multiple protrusions pointing to the ventricular surface of the heart [42]. A subset of mesothelial cells detaches from the epicardium, migrates in the subepicardial space and undergoes epithelial-mesenchymal transition, generating a population of epicardially derived cells (EPDCs). These cells contribute to several cell lineages within the developing heart [19].

Recent studies have observed the preservation of multipotency of adult epicardial cells *in vitro* [21, 22], suggesting that the EPDCs form myocardial precursor cells, that mediate regeneration of adult heart in physiological and pathological conditions. In the study contemporary with ours, Capogrossi et al. [43] identified CD117-positive cells in the subepicardial region of adult hearts. Immunofluorescence analysis revealed that some subepicardial CD117-positive cells expressed the early marker of cardiomyocyte differentiation Nkx2.5 and the cardiac transcription factor GATA4, and displayed the functional characteristics of endothelial cells (Ac-LDL-Dil uptake). The number of these cells in the murine heart increased 3 days after an experimental infarction. However, the mechanistic connection between epicardial cells and CD117-positive cells was not shown experimentally.

Unlike most studies dedicated to the epithelial-mesenchymal transition of adult epicardial cells that described this phenomenon *in vitro* as spontaneous [22] or in which fibroblast-like shaped cells were isolated

directly from subepicardium [43], our study provides direct evidence of epicardial origin of CD117-positive cells in the adult human heart. The epithelial sheet formed *in vitro* only in the presence of extracellular matrix typical of adult human heart. This fact underscores the role of subepicardial space, rich in extracellular matrix fibers, proteoglycans and glycoproteins, in the preservation of epicardium integrity. On the other hand, the subepicardium also accumulates growth factors, such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and transforming growth factors (TGF), that were shown to induce the epithelial-mesenchymal transition in the variety of cell types [17] and, specifically, to regulate the development of epicardially-derived cells. In fact, soon after the addition of HGF, we observed that the epicardial cells forming a compact monolayer *in vitro* changed to CD117-positive spindle-like shaped small cells with numerous lamellipodia and motile properties. This finding, when confirmed *in vivo*, could cast new light on the origin of multipotent cardiac primitive CD117-positive cells in the adult human heart.

6. Conclusions

The findings of the present study indicate that normal adult human heart and pathological heart with ischemic cardiomyopathy differ in terms of CD117-positive cells number and laminin isoforms and integrin α_6 subunit expression. While in the normal heart laminin-1 is barely present in the left ventricle and most of CD117-positive cells do not express α_6 integrin, in the pathological conditions the expression of laminin-1 is significantly higher and α_6 integrin-positive cardiac primitive cells predominate. Moreover, the number of CD117-positive cells is strikingly higher in the pathological conditions. Remarkably, these cardiac primitive cells are located in the subepicardium of the ischemic heart. The induction of epithelial-mesenchymal transition in the epicardial cells of adult human heart *in vitro* provides the connection between these cells and CD117-positive cells, known to be able to give rise to cardiomyocytes, smooth muscle and endothelial cells.

As is the case with most tissues and organs with self-renewing capacity, among which skin, liver and intestine, ischemia leads inevitably to the cell necrosis and scar formation. The most common modality of heart ischemia is associated with a subendocardial infarction and, more rarely, full wall thickness necrosis. The epicardium and subepicardial region usually retain a relatively better blood supply. When these facts are taken into consideration, the possible epicardial origin and subepicardial localization

seem advantageous to cardiac primitive cells, constituting the “niche” that supports their survival and proliferation, enabling their survival and migration in the pathological conditions.

In organs with regenerative capacity, the physiological cell turnover enables the preservation of tissue structure and function, inasmuch as old or dead cells are replaced with the new and better functioning ones. In pathological conditions, the bigger the damage, the bigger resources must be involved in the healing process and it has been already suggested that organ regeneration may require molecular reprogramming and reactivation of the mechanisms operative during organogenesis [41]. The epithelial–mesenchymal transition and the generation of CD117-positive cells, as well as the expression of laminin α_1 chain in the adult pathological heart, followed by different expression of integrin subunits on cardiac cells, may represent such process, in which an extensive damage and chronic pathological conditions activate a regenerative response involving all, cardiac primitive cells, extracellular matrix proteins and their receptors.

In the foregoing discussion I have attempted to examine the links between different laminin and integrin isoforms expression and CD117-positive cells localization in the adult human heart. On balance, we suggest a model of cardiac regeneration in which the signals from subepicardial space, among which the acquisition of laminin-1 expression, activate the epithelial-mesenchymal transition of epicardial cells, leading to the population of subepicardium with CD117-positive cells. The interaction of

integrin α_6 on the cell membrane and laminin-1 in the extracellular matrix supports the survival of cells, protecting them from apoptosis, and stimulates their migration towards a damaged muscle. Once within the cardiac wall, primitive cells differentiate and lose gradually the expression of the stem cell marker (CD117). They anchorage to the cardiac stroma rich in fibronectin by means of integrin α_4 and become functionally incorporated within the cardiac muscle syncytium bordered by laminin-2. Our results furnish the intriguing, still indirect, evidence that these processes are remarkably activated in the ischemic cardiomyopathy. An experimental model allowing cardiac CD117-positive cells tracing *in vivo* is necessary to verify the proposed model emerging from the examination of heart sections and *in vitro* experiments.

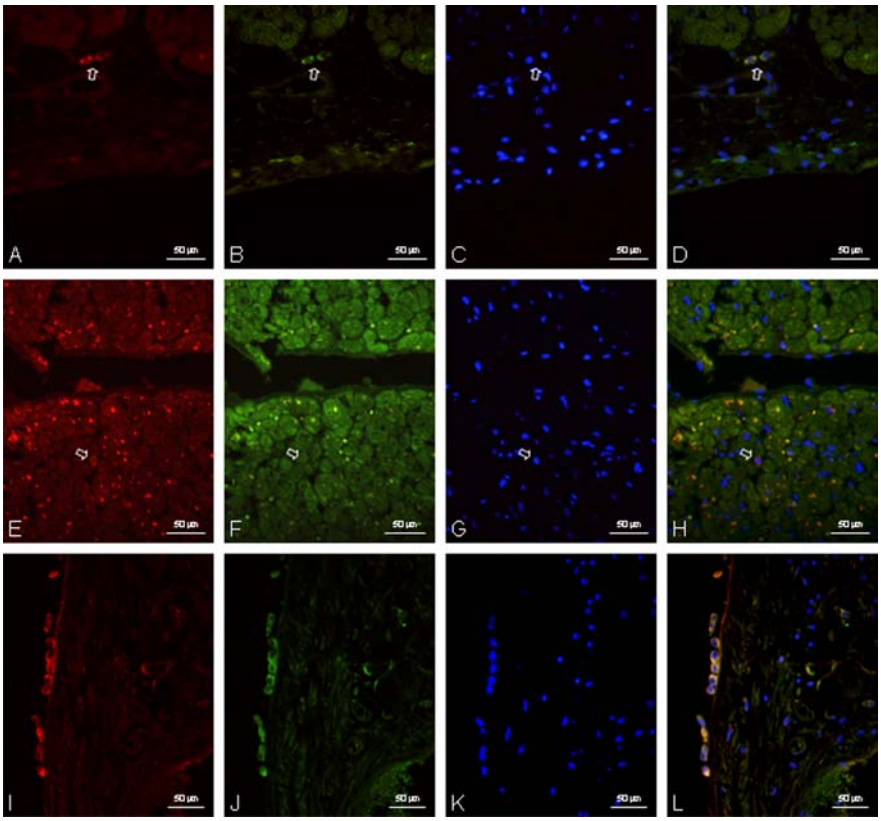

Figure 1.
CD117-positive cells in the subepicardium and myocardium of the adult human heart detected by immunofluorescence.

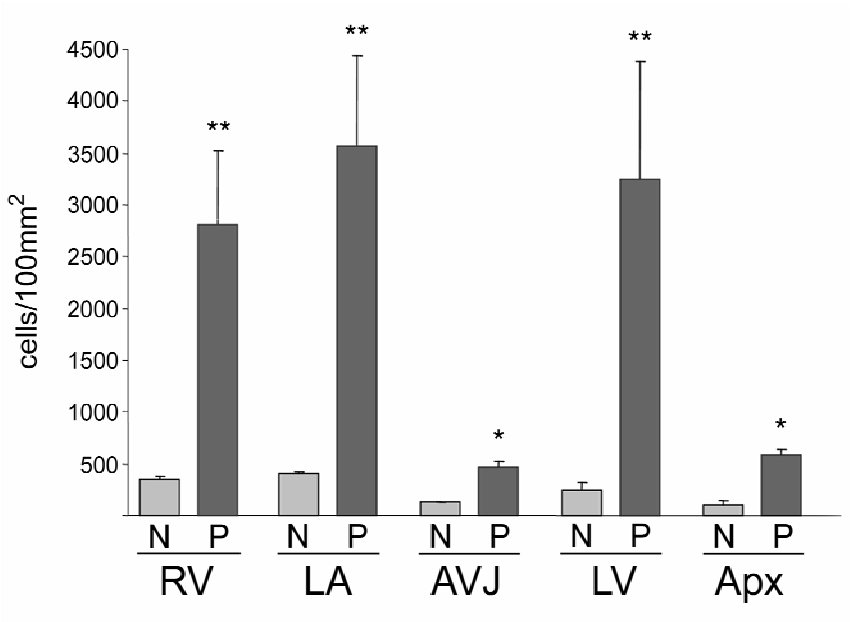

Figure 2.
Distribution of CD117-positive cells between different heart regions.

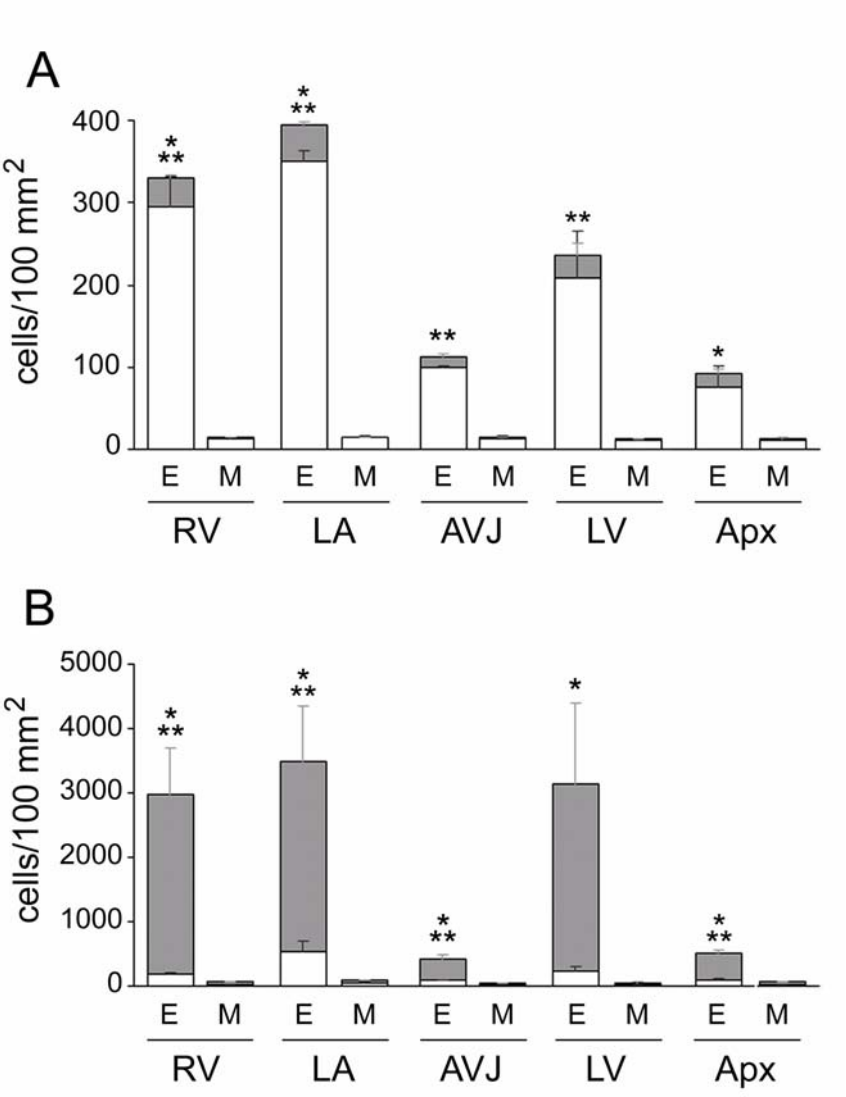

Figure 3.
Distribution of CD117-positive cardiac cells and expression of α_6 integrin between different cardiac tissue layers.

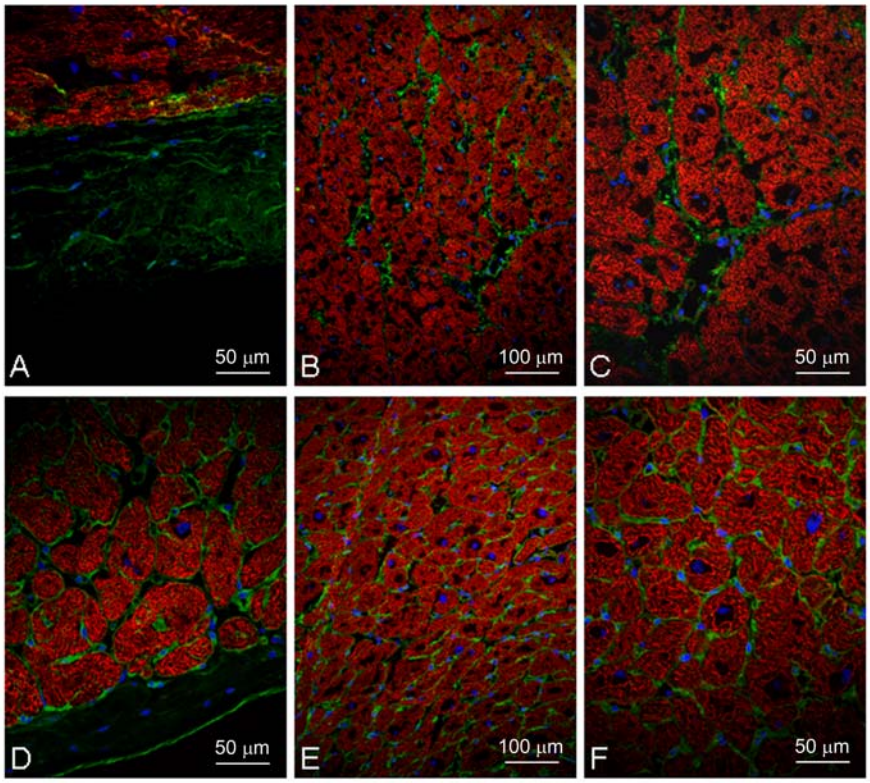

Figure 4.
Pattern of laminin-1 and -2 distribution in the pathological adult human heart tissue sections.

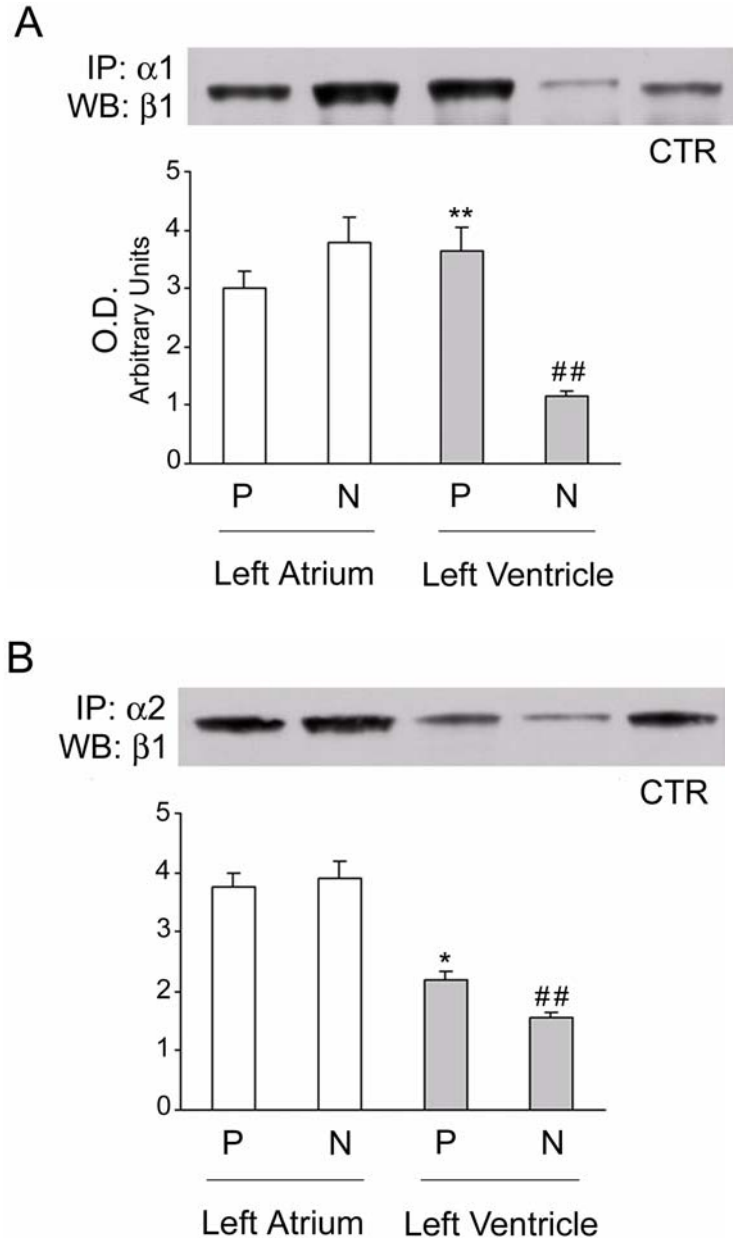

Figure 5.
Expression of laminin-1 and -2 in different heart regions.

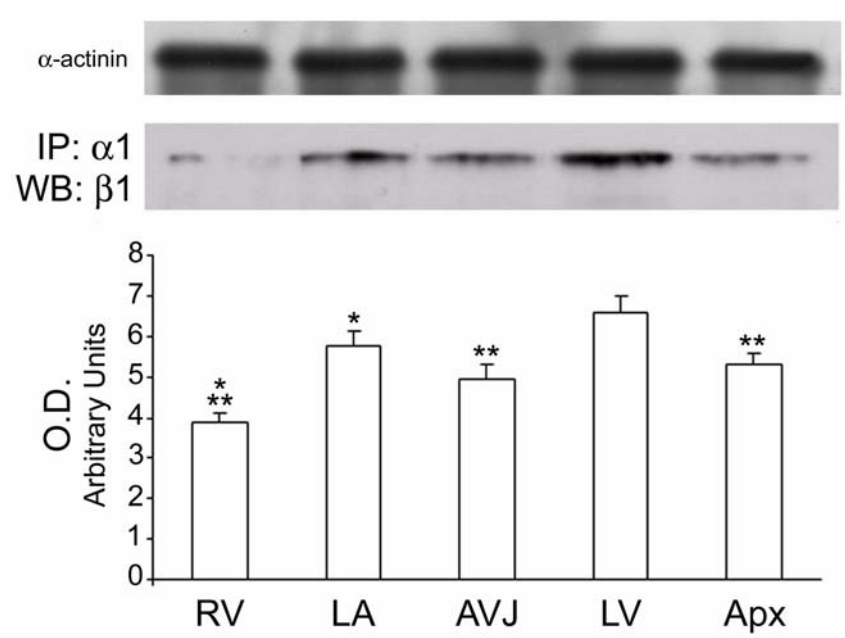

Figure 6.
Expression of laminin-1 in the adult human heart with ischemic cardiomyopathy.

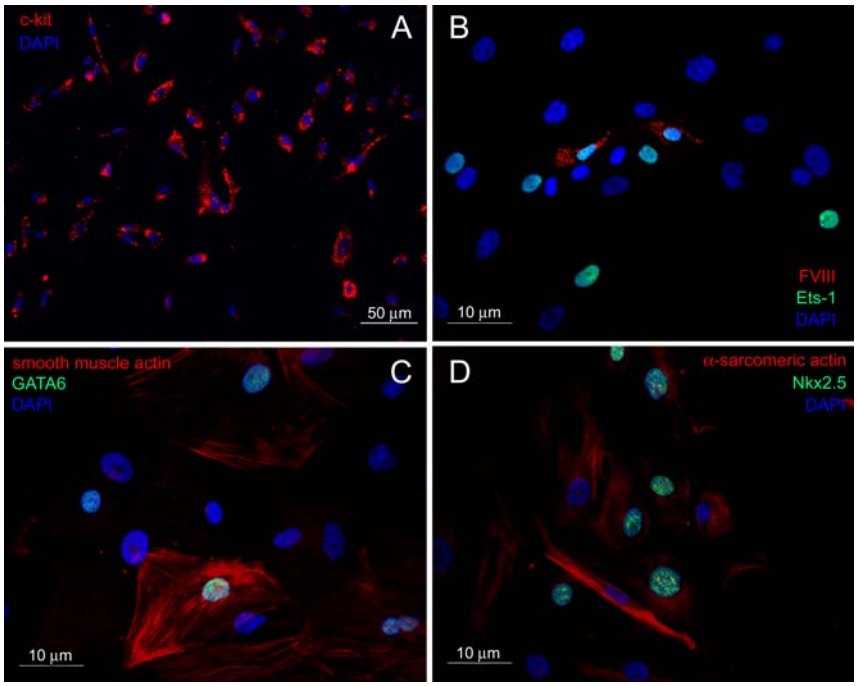

Figure 7.
CD117-positive primitive cells *in vitro*.

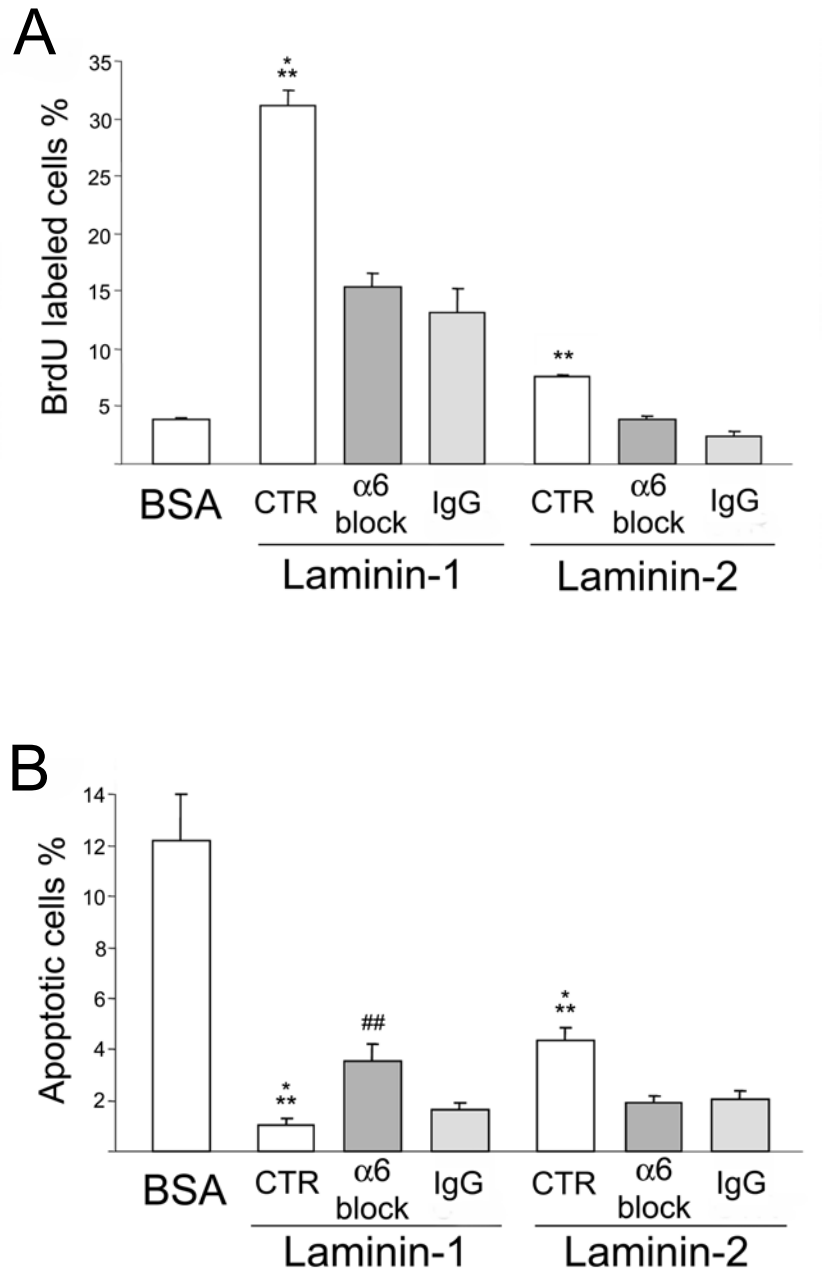

Figure 8.
Effects of laminin-1 and -2 on proliferation and apoptosis of cardiac CD117-positive cells *in vitro.*

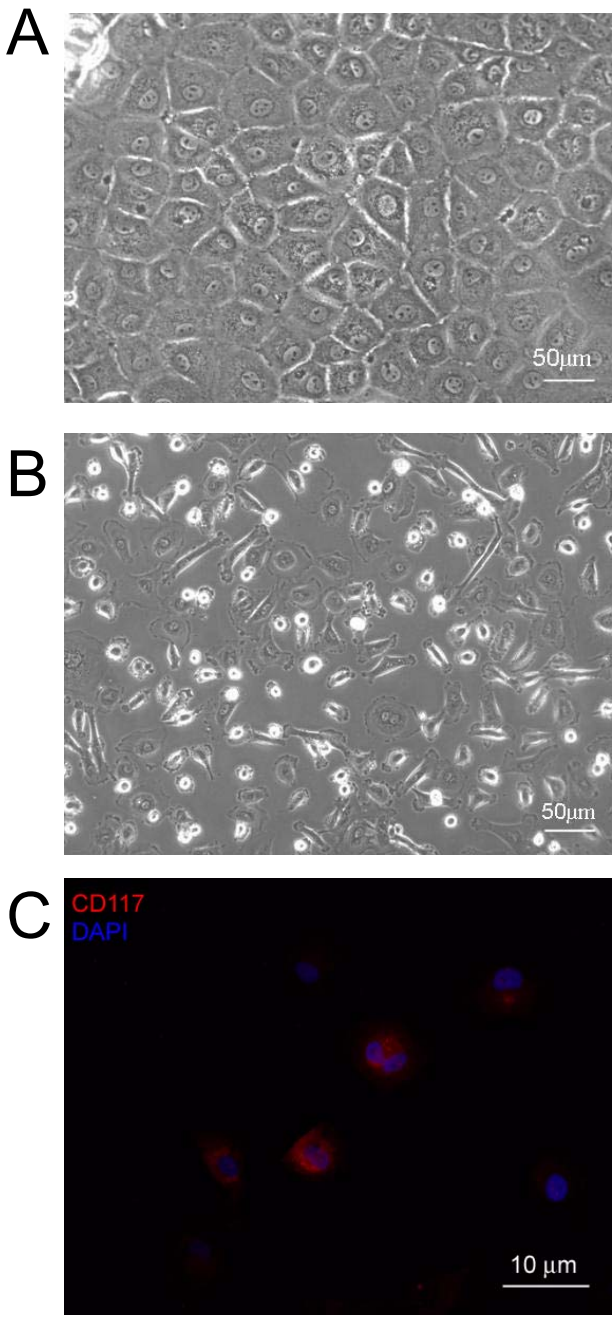

Figure 9.
Epithelial-mesenchymal transition *in vitro*.

Figure legends

Figure 1. CD117-positive cells in the subepicardium and myocardium of the adult human heart detected by immunofluorescence. Examples of normal heart sections staining are presented. **Top row**: Two CD117-positive cells (arrow) within the subepicardium (A, red) express α_6 integrin subunit (B, green). **Middle row**: CD117-positive cell (arrow) within the myocardium (E, red) is α_6 integrin-negative (F). **Bottom row**: Fibronectin lines epithelial cells within the epicardium (I, red); all cells forming the epicardium are α_4 integrin-positive (J, green) and co-localize with fibronectin. The nuclei of cells were counterstained with DAPI (C, G, K, blue). Panels D, H, L result from the overlay of three separate images from every row. Scale bar: 50 μm.

Figure 2. Distribution of CD117-positive cells between different heart regions. CD117-positive cells in the sections from the right ventricle (RV), left atrium (LA), atrioventicular junction (AVJ), left ventricle (LV) and apex (Apx) of the normal (N) and pathological (P) hearts were visualized by immunofluorescence and quantified. The bars correspond to the mean±SEM of CD117-positive cells number in 100 mm^2. With respect to the same regions of the normal hearts (n=4 for every region), these cells are 8.7-fold more numerous in LA (n=5), 3.6-fold in AVJ (n=4), 13-fold in LV (n=4), 8.1-fold in RV (n=5) and 5.4-fold in Apx (n=5) of the hearts with ischemic cardiomyopathy. *p<0.05, **p<0.001 normal versus pathological.

Figure 3. Distribution of CD117-positive cardiac cells and expression of α_6 integrin between different cardiac tissue layers. CD117-positive α_6 integrin-positive (gray bar) and α_6 integrin-negative (white bar) cells in the epicardium/subepicardium (E) and myocardium (M) of the right ventricle (RV), left atrium (LA), atrioventricular junction (AVJ), left ventricle (LV) and apex (Apx) were visualized by immunofluorescence and quantified. The bars correspond to the mean±SEM of CD117-positive cells number in 100 mm^2. **A:** In the normal hearts CD117-positive cells are localized mainly in the epicardium of the atrium and right ventricle; the fraction of α_6 integrin-expressing cells is identical in the subepicardium and myocardium. **B:** In the pathological hearts CD117-positive cells are significantly more numerous (note the difference in the scale), with their highest number in the subepicardium of LA, LV and RV; the fraction of α_6 integrin-positive cells is from 2 to 4-fold higher than in the normal hearts and 2-fold higher in the subepicardium than in the myocardium. *p<0.05, **p<0.001, ***p<0.0001 CD117-positive cells in the subepicardium versus myocardium.

Figure 4. Pattern of laminin-1 and -2 distribution in the pathological adult human heart tissue sections. The presence of laminin-1 and merosins (laminin-2 and -4) in the hearts with chronic ischemic cardiomyopathy was detected by immunofluorescent labeling of heart sections with specific antibodies against α_1 and α_2 laminin chains (green). Antibody against α-sarcomeric actin was used to stain cardiomyocytes (red); the nuclei of cells

were counterstained with DAPI (blue). **A:** Laminin-1 fills subepicardium with a meshed network; a few branches spread between epicardium and myocardium are present. **B, C:** Granular-like pattern of laminin-1 immunofluorescence can be observed within the myocardium, where laminin-1 fills interstitial spaces and clusters round the cardiac cells. **D-F:** Merosins line epicardium and form a conspicuous network corresponding to the basement membrane of the cells in the myocardium.

Figure 5. Expression of laminin-1 and -2 in different heart regions. The expression of laminin-1 (A) and laminin-2 (B) isoforms in the adult human heart was detected by immunoprecipitation (IP: α_1 or α_2) followed by western blotting (WB: β_1) of the proteins from normal (N) and pathological hearts (P). Representative results with bands of 220 kDa corresponding to $\alpha_1\beta_1$ or $\alpha_2\beta_1$ laminin chains are shown above the bars indicating their mean±SEM optical density. CTR indicates positive control for western blot (purified laminin-1 or laminin-2). *p<0.05, **p<0.001 pathological versus normal within the same heart region, ## p<0.001 left ventricle versus atrium within the same heart.

Figure 6. The expression of laminin-1 in the adult human heart with ischemic cardiomyopathy was detected by immunoprecipitation (IP: α_1) followed by western blotting (WB: β_1) of the proteins from right ventricle (RV), left atrium (LA), atrioventricular junction (AVJ), left ventricle (LV)

and apex (A). Western blot of α–actinin (110 kDa) served as a control of equal protein amount in the solution used for immunoprecipitation. Representative result with bands of 220 kDa corresponding to $\alpha_1\beta_1$ laminin chains is shown above the bars indicating mean±SEM optical density. *p<0.05, **p<0.001, ***p<0.0001 versus LV.

Figure 7. CD117-positive primitive cells *in vitro*. CD117-positive cells were isolated from the fragments of left ventricular myocardium of adult human hearts by enzymatic dissociation and immunomagnetic separation. **A:** The efficacy of separation was confirmed by immunofluorescent labeling of CD117 antigen and reached 98%. **B-E:** The presence of cells expressing nuclear and cytoplasmic markers of endothelial (Ets-1 and factor VIII), smooth muscle (GATA6 and smooth muscle actin) and cardiomyocyte (Nkx2.5 and α-sarcomeric actin) cell lineages among CD117-positive cells in culture was evidentiated by immunofluorescence.

Figure 8. Effects of laminin-1 and -2 on proliferation and apoptosis of cardiac CD117-positive cells *in vitro*. The bars correspond to the mean±SEM percentage of cells with BrdU incorporation (A) or the percentage of apoptotic cells (B). CD117-positive cells were plated on laminin-1 or laminin-2 coated dishes after incubation with α_6 integrin function-blocking antibody (α_6 block), isotype-matched control antibody (IgG) or without antibody pretreatment (CTR). **A**: Both laminin-1 and -2

stimulate proliferation of CD117-positive cells *in vitro*. The percentage of BrdU incorporating cells in the presence of laminin-1 is 4.5-fold higher with respect to laminin-2. However, this effect is not related to α_6 integrin expression, as both α_6 integrin function-blocking antibody and isotype-matched non specific antibody reduce proliferation rate in the same manner. **B:** Laminin-1 and -2 protect CD117-positive cells from apoptosis. Moreover, the percentage of apoptotic cells is 4-fold lower in the presence of laminin-1 with respect to laminin-2. The anti-apoptotic effect of laminin-1 is specifically abolished in the presence of α_6 integrin function-blocking antibody. ***p*<0.001, ****p*<0.0001 CTR versus BSA, ##*p*<0.001 α_6 block versus IgG.

Figure 9. Epithelial-mesenchymal transition *in vitro*. **A**: Phase-contrast images show the epithelial sheet obtained from adult human epicardium placed on culture dish in the presence of extracellular matrix typical of pathological heart. **B:** After the addition of HGF, the epithelial cells disaggregated, giving origin to small spindle-like shaped cells with motile properties. **C:** These cells express CD117 antigen (red fluorescence). The nuclei of cells were counterstained with DAPI (blue fluorescence).

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